

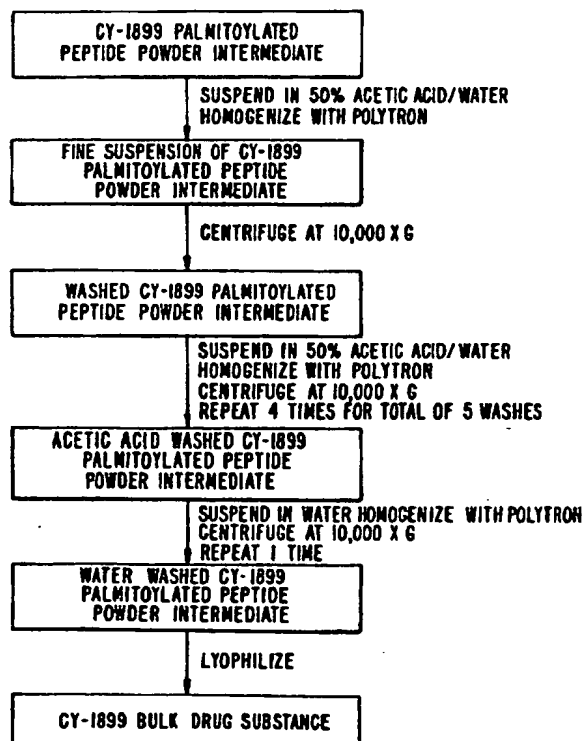


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<b>(21) International Application Number:</b> PCT/US96/09579 <b>(22) International Filing Date:</b> 5 June 1996 (05.06.96) <b>(30) Priority Data:</b> 08/483,196      7 June 1995 (07.06.95)      US <b>(71) Applicant:</b> CYTEL CORPORATION [US/US]; 3525 John Hopkins Court, San Diego, CA 92121 (US). <b>(72) Inventors:</b> KUBO, Ralph, T.; 12635 Futura Street, San Diego, CA 92130 (US). ARRHENIUS, Thomas; 3909 San Augustine Way, San Diego, CA 92130 (US). <b>(74) Agents:</b> BASTIAN, Kevin, L. et al.; Townsend and Townsend and Crew L.L.P., Two Embarcadero Center, San Francisco, CA 94111-3834 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** MANUFACTURE AND PURIFICATION OF PEPTIDES**(57) Abstract**

The present invention provides methods for preparing purified synthetic lipopeptides. The methods comprise contacting a composition comprising the synthetic lipopeptide and various contaminants with an aqueous solution, such that impurities are selectively dissolved.



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MANUFACTURE AND PURIFICATION  
OF PEPTIDES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to improved methods for the manufacture and purification of peptides. In particular, it relates to methods of purifying lipopeptides useful as peptide-based therapeutics.

Background

Cytotoxic T lymphocytes (CTLs) are important in the elimination of unwanted cells such as viral infected or neoplastic cells. CTLs specifically recognize and kill cells which are infected by a virus or which express certain tumor related antigens.

The anti-viral or anti-tumor activity of CTL is the result of a series of complex molecular events. After cellular processing of proteins in the cytoplasm of the virally infected cells or tumor cells, small peptides are transported to the endoplasmic reticulum, where they bind to newly synthesized major histocompatibility gene complex (MHC) class I molecules. MHC/peptide complexes are then exported to the surface of the cell, where they are recognized by antigen-specific Class I-restricted CTL.

Immunologists have long been interested in the induction of specific CTL responses for the treatment of disease. For instance, *in vivo* induction of anti-viral CTL may be achieved by injection of live viruses. This procedure, however, bears two major disadvantages: 1) risk of induction of some form of the disease, and 2) lack of direct control over CTL epitope specificity.

Since the discovery that short synthetic peptides can mimic the endogenous antigens recognized by CTL, much effort has been devoted to the development of synthetic peptide immunogens for the treatment of a number of diseases. Inexpensive and efficient methods for the manufacture of human peptide-based vaccines or therapeutic agents are particularly desirable. The present invention provides these and other advantages.

## SUMMARY OF THE INVENTION

The present invention provides methods for preparing purified synthetic lipopeptides. The methods comprise contacting a composition comprising the synthetic lipopeptide with an aqueous solution, such that impurities are selectively dissolved. The lipopeptide is then separated, typically by centrifugation, from the aqueous solution to obtain a purified lipopeptide. The aqueous solution typically comprises an organic acid, such as acetic acid. A preferred aqueous solution is 50% acetic acid/50% water. The steps of contacting the lipopeptide with the aqueous solution may be repeated, typically about 5 times.

In some embodiments, the lipopeptide comprises two lipid residues. The lipid residue may be any lipid moiety, an exemplary lipid is a palmitoyl residue. The lipopeptide may be of any length, but typically comprises between about 10 and about 35 amino acid residues.

The lipopeptide is typically an immunogenic peptide and thus comprises a CTL epitope, such as one from the hepatitis B virus core antigen. An exemplary CTL epitope is Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val. The lipopeptide may also comprise a T helper epitope. An exemplary T helper epitope is Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu. A preferred lipopeptide is (PAM)<sub>2</sub>-Lys-Ser-Ser-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val.

The invention also provides compositions comprising the purified lipopeptides of the invention. The compositions consist essentially of DMSO, TFA and a lipopeptide having the formula:

(PAM)<sub>2</sub>-Lys-Ser-Ser-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val.

## Definitions

The term "peptide" is used interchangeably with "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids.

An "immunogenic peptide" is a peptide that will bind an MHC molecule and induce a CTL response. The immunogenic peptides of the invention are less than about 50

residues in length and usually consist of between about 10 and about 45 residues, preferably between about 20 and about 35 residues.

In preferred embodiments the immunogenic peptides comprise a peptide comprising a CTL epitope covalently linked to a T helper peptides. Particularly preferred CTL inducing peptides/T helper conjugates are linked by a spacer molecule.

A "lipopeptide" as used herein refers to a peptide, preferably an immunogenic peptide, covalently linked to a lipid residue. A lipid residue is any residue derived from an organic molecule, including, fats, oils, waxes, and the like, which is relatively insoluble in water but soluble in common organic nonpolar solvents.

An impurity or contaminant is "selectively dissolved" in an aqueous solution as compared to the lipopeptides of the invention if the impurity is essentially completely in solution, such that after separation of the lipopeptide from the solution, the contaminant is not detectable in the lipopeptide composition by standard techniques such as HPLC. In particular, a chromatogram as shown in Figure 3 (using the conditions described there) shows the degree of purity of the final lipopeptide product. Typically, the contaminants will be byproducts of peptide synthesis, such as uncompleted peptide chains. The differential solubility of the uncompleted chains is enhanced by the capping procedure used during peptide synthesis.

A "purified peptide" or "purified lipopeptide" refers to material which is free from contaminants such as uncompleted peptide chains as ascertained by standard techniques such as HPLC described above. In particular, the purified peptides or lipopeptides of the invention are sufficiently free of contaminating material to suitable for administration to humans.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow chart showing the process steps in the purification of a lipopeptide of the invention, CY-1899.

Figure 2 shows a typical HPLC profile of CY-1899 Palmitoylated Peptide Powder (unpurified). The major peak (approximately 31 minutes) corresponds to CY-1899. Several impurities can also be observed in the chromatogram. The chromatographic conditions are as follows: Sample concentration - 10 mg/mL in DMSO + 0.1% TFA. Injection volume - 5  $\mu$ L. column - Zorbax 300SB-CN, 5  $\mu$ M, 30 nm pore, 150 mm 4.6mm. Mobile Phase - A: 20% Acetonitrile, 79.9% water, 0.1%

Trifluoroacetic acid, B: 99.9% Acetonitrile, 0.1% trifluoroacetic acid. Column temperature 60°C. Gradient - 5% to 100% B in 45 minutes. Flow rate - 1 mL/min. Detection - UV at 214 nm.

Figure 3 shows the results of HPLC chromatography of CY-1899 Bulk Drug Substance after purification. CY-1899 Bulk Drug Substance was dissolved in DMSO/0.1% TFA at 5mg/mL. The sample (1 $\mu$ l) was applied to a Zorbax 300SB-CN HPLC column (50 mm X 4.6mm). The column temperature was 60°C, the flow-rate was 1.0 mL/min, and the eluate was monitored at 214 nm. Buffer A was 20% acetonitrile, 80% water and 0.1% trifluoroacetic acid. Buffer B was 100% acetonitrile containing 0.095% trifluoroacetic acid. The initial conditions were 95% buffer A and 5% buffer B. The gradient was 5% buffer B isocratic for 10 minutes, from 5% B to 100% B over 45 minutes followed by isocratic 100% B for 10 minutes. CY-1899 eluted at approximately 31 minutes.

Figure 4 shows the results of Mass Spectral Analysis of CY-1899 Contaminants. A mixture of several impurities was obtained by fractionation by semi-preparative HPLC chromatography of Palmitoylated Peptide Powder before purification of CY-1899. High resolution ion spray mass spectrometric analysis of this sample indicated the presence of at least 3 compounds. Each of these impurities has a molecular ion designated as M, M<sup>1</sup>, M<sup>2</sup> similar in mass to that of CY-1899 (MH<sup>+</sup> = 3742), suggesting that these impurities are closely related to CY-1899. The structures have not yet been elucidated. The sodium adducts of the molecular ions M, M<sup>1</sup> and M<sup>2</sup> are designated as M-Na, M<sup>1</sup>-Na, and M<sup>2</sup>-Na.

Figure 5 shows mass spectrometric analysis of CY-1899. High resolution FAB<sup>+</sup> mass spectrometric analysis prior to purification of CY-1899 indicates the presence of a number of impurities, several of which can be identified as non-palmitoylated, acetylated peptides, which arise as a result of the capping procedure used during the synthesis. The tentative assignments are as follows:

molecular ion 3742 CY-1899

molecular ion 3093 Ac-SQYIKANSKFIGITEAA AFLPSDFFPSV

30 molecular ion 2877 Ac-YIKANSKFIGITEAA AFLPSDFFPSV

molecular ion 2715 Ac-IKANSKFIGITEAA AFLPSDFFPSV

molecular ion 2602 Ac-KANSKFIGITEAA AFLPSDFFPSV

molecular ion 2287 Ac-SKFIGITEAA AFLPSDFFPSV

molecular ion 1753 Ac-ITEAAAFLLPSDFFPSV

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The present invention provides improved methods for the production of peptide-based vaccines and therapeutic agents. The peptides of the invention comprise T cell epitopes from any desired antigen, for which the amino acid sequence of the antigen is known.

Epitopes derived from a number of potential target proteins can be used. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, 10 melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens. For a description of the identification of peptides comprising epitopes derived from particular antigens see WO 95/01000 and WO 94/02353. Particularly preferred peptides for the treatment of HBV are disclosed in WO 92/07218.

15 The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown, are in the form they would 20 assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids is represented by a lower case single letter or a lower case three letter symbol. Glycine has 25 no asymmetric carbon atom and is simply referred to as "Gly" or G.

### Immunogenic peptides

The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology. As described in detail below, the peptides are preferably prepared using 30 synthetic methods. Although the peptide will preferably be free of other naturally occurring host cell proteins and fragments thereof, the peptides are synthetically conjugated to lipid residues as described below. The polypeptides or peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either

free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, the peptides may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding.

The peptides can also be modified by extending or decreasing the compound's amino acid sequence, e.g., by the addition or deletion of amino acids. The peptides or analogs of the invention can also be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, e.g., those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins, such as L- $\alpha$ -amino acids, or their D-isomers, but may include non-natural amino acids as well, such as  $\beta$ - $\gamma$ - $\delta$ -amino acids, as well as many derivatives of L- $\alpha$ -amino acids.

Amino acid substitutions are typically of single residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final peptide. Substitutional variants are those in which at least one residue of a peptide has been removed and a different residue inserted in its place.

Substantial changes in function (e.g., affinity for MHC molecules or T cell receptors) are made by selecting substitutions that are less conservative, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in peptide properties will be those in which (a) hydrophilic residue, e.g. seryl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or



(c) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The peptides of the invention can be combined via linkage to form polymers (multimers), or can be formulated in a composition without linkage, as an admixture.

5 Where the same peptide is linked to itself, thereby forming a homopolymer, a plurality of repeating epitopic units are presented. In addition to covalent linkages, noncovalent linkages capable of forming intermolecular and intrastructural bonds are also contemplated. Linkages for homo- or hetero-polymers or for coupling to carriers can be provided in a variety of ways well known to those of skill in the art.

### 10 T helper peptides

In another aspect the immunogenic peptides of the invention are combined or coupled with other peptides which present T-helper cell epitopes, i.e., T helper peptides comprising six to thirty amino acids containing a T helper epitope which from the  
15 envelope, core or other immunogenic protein or derivative thereof, stimulate T cells that cooperate in the induction of cytotoxic T cells to the target antigen. Compositions of T-helper peptides and CTL peptides thereby enhance an individual's immunity by providing cell-mediated immunity and protective antibodies. T helper epitopes are provided by peptides from, for example, tetanus toxoid 830-843, malaria circumsporozoite 382-398,  
20 malaria circumsporozoite 378-398, ovalbumin 323-336, and the influenza epitope 307-319 (see, WO 95/01000, WO 94/02353 and WO 92/07218, *supra*).

In addition, pan DR helper peptides can be used as the helper peptides. Pan DR peptides are those recognized by a broad pattern of DR alleles. For a description of the production and use of pan DR peptides, see, WO 94/10368.

25 In preferred embodiments the CTL inducing peptides of the invention are covalently linked to the T helper peptides. Particularly preferred CTL inducing peptides/T helper conjugates are linked by a spacer molecule. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are  
30 substantially uncharged under physiological conditions and may have linear or branched side chains. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. In certain preferred embodiments herein the neutral spacer is Ala. It will be understood that the optionally

present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. Preferred exemplary spacers are homo-oligomers of Ala. When present, the spacer will usually be at least one or two residues, more usually three to six residues.

## 5 Lipid residue

The peptides of the invention comprise at least one lipid residue which assists in priming CTL. Lipids have been identified as agents capable of assisting the priming CTL *in vivo* against viral antigens. Thus various alkanoyl ( $C_1$ - $C_{20}$ ) lipids can be linked to the peptides via one or more linking residues. For example, palmitic acid  
10 residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen  
15 comprises palmitic acid (referred to as PAM) attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine ( $P_3$ CSS) can be used to prime virus  
20 specific CTL when covalently attached to an appropriate peptide. See, Deres et al., *Nature* 342:561-564 (1989). Peptides of the invention can be coupled to  $P_3$ CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with  $P_3$ CSS conjugated to a peptide which displays an appropriate epitope, the  
25 two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

## Preparation and purification of lipopeptides

The lipopeptides of the invention can be prepared in a wide variety of ways.  
30 Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or

transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982).

5 As noted above, the preferred method of making the peptides of the invention is through synthetic means. Solid phase peptide synthesis in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is the preferred method for preparing the peptides of the present invention. Techniques for solid phase synthesis  
10 are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis*; pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.*, Merrifield, et al. *J. Am. Chem. Soc.* 85, 2149-2156 (1963), and Gross and Meienhofer, eds. Academic press, N.Y., 1980 and Stewart et al., *Solid Phase Peptide Synthesis*, 2nd ed. Pierce Chem. Co., Rockford, Ill. (1984). Solid phase synthesis is  
15 most easily accomplished with commercially available peptide synthesizers utilizing, for example, Fmoc or TboC chemistry.

As explained in detail below, the lipopeptides of the invention are produced by assembly of the lipopeptide from C-terminus to N-terminus on standard supports, Fmoc chemistry. After each residue is added to the growing oligopeptide chain, the chain is  
20 capped, according to standard procedures, e.g., by reaction with acetic anhydride. This will ensure that any resin bound peptide chain that was not coupled with the last amino acid will be terminated. These terminated chains will then be removed at the final purification step of the lipopeptide due to their different physical properties from the lipopeptide in aqueous solutions.

25 In the exemplified embodiment, synthesis and purification of a preferred embodiment, referred to here as CY1899, is described in detail. One of skill will recognize that other lipopeptides can be prepared in essentially the same manner. For instance, the methods have also been shown to be effective for other lipopeptides such as (PAM)<sub>2</sub>KSSAKXVAAWTLKAAAAAGIGILTV, where X is L-cyclohexylalanine.

30 N-terminal lipidation is also carried out on the resin. Following assembly of the fully protected peptide, the desired product is cleaved from the resin with concomitant deprotection of the side chain protecting groups. Upon completion of the coupling cycles

and cleavage from the solid support with trifluoroacetic acid (TFA), the lipopeptide is precipitated with diethyl ether.

Purification of lipopeptides of the invention is accomplished by suspending the lipopeptide in a polar solvent, typically aqueous solutions, such as water, aqueous solutions of weak organic acids, phosphate buffered saline (PBS), and normal saline (0.9% sodium chloride). The lipopeptides are very slightly soluble in dimethyl sulfoxide (DMSO) and anhydrous methanol, but are sparingly soluble in these solvents when trifluoroacetic acid (TFA) (0.1%-0.5%) is added. (Solubility descriptions are in accordance with USP definitions). Thus, the relative insolubility of the lipopeptides in polar aqueous solutions, as compared to contaminants, provides a particularly convenient method for preparing purified lipopeptides. One of skill will recognize that any number of polar solvents can be used for this purpose, and the particular solvent is not critical, so long as the contaminants are much more soluble in the solution than the lipopeptides.

Although any solution may be used for this purpose, an aqueous solution comprising a weak organic acid, such as acetic acid, is typically used. A preferred solute is 50% acetic acid/water. The lipopeptide, unlike other reaction byproducts such as incomplete oligopeptide chains, is not soluble in the aqueous solution and is therefore recovered in purified form by separating it from the solution. The lipopeptide can be recovered according to any method commonly used to recover insoluble material from a solution, typically by centrifugation.

The process may be repeated until the desired purity is obtained. In a typical embodiment, this will be include between about 2 washings and about 10 washings. Washing a total of about 5 times is usually sufficient to prepare lipopeptides of suitable purity. After repeated washing the lipopeptide is washed twice with water and the final pellet is resuspended in water and lyophilized. The lipopeptide is then dissolved in an appropriate solvent, such as DMSO.

### **Pharmaceutical compositions**

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent viral infection and cancer. Examples of diseases which can be treated using the immunogenic peptides of the invention include prostate cancer, hepatitis B,

hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV and condyloma acuminatum.

For pharmaceutical compositions, the immunogenic peptides of the invention are administered to an individual already suffering from cancer or infected with the virus of interest. Those in the incubation phase or the acute phase of infection can be treated with the immunogenic peptides separately or in conjunction with other treatments, as appropriate. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to cure or at least partially arrest symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0  $\mu\text{g}$  to about 5000  $\mu\text{g}$  of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0  $\mu\text{g}$  to about 1000  $\mu\text{g}$  of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of viral infection or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for

instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide compositions can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers.

5 It is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic infection, a representative dose is in the range of about 1.0  $\mu\text{g}$  to about 5000  $\mu\text{g}$ , preferably about 5  $\mu\text{g}$  to 1000  $\mu\text{g}$  for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to  
10 four weeks, may be required, possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic treatment are intended for  
15 parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier with an appropriate  
20 solvent such as DMSO/TFA. A variety of aqueous carriers may be used, e.g., water, buffered water, PBS, 0.3% glycine, hyaluronic acid and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium  
25 chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of CTL stimulatory peptides of the invention in the final pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 0.1% to 0.5%, to as much as 10% to 15% or more by weight, and will  
30 be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The following examples are offered to illustrate, but not limit, the invention.

## Examples

### I. Description, Physical and Chemical Characteristics

The lipopeptide CY-1899 is a construct consisting of thirty L-amino acid residues and two palmitic acid residues. The amino acids are covalently linked in a linear fashion through amide bonds. The two palmitoyl (PAM) residues are linked to the N-terminus amino acid through amide bonds to the  $\alpha$  and  $\epsilon$  amino groups of lysine.

The composition of CY-1899 is as follows:

(PAM)<sub>2</sub>-Lys-Ser-Ser-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val

The ten amino acid sequence from the phenylalanine at residue 21 through the C-terminus valine forms the CTL epitope from hepatitis B virus core antigen. A three amino acid linker consisting of alanines is used to connect the CTL epitope to the next sequence which is a fourteen amino acid sequence of tetanus toxoid that serves as a T cell helper epitope. This is followed by a three amino acid linker terminating in lysine. The N-terminus lysine provides two amino groups to which palmitic acid is attached. In this way the construct contains three moieties: CTL epitope, T-helper epitope, and lipid group, covalently coupled together to give active CY-1899.

The lipid groups provide for particular physical properties of CY-1899. It is insoluble in water and soluble in methanol. This property allows for purification of CY-1899 from water soluble impurities by the addition of water. CY-1899 is also insoluble in 50% acetic acid/water. The preferred purification scheme employs multiple washings in 50% acetic acid/water to remove soluble peptide and non-peptide contaminants from the crude peptide intermediate. CY-1899 is practically insoluble in water, phosphate buffered saline (PBS), and normal saline (0.9% sodium chloride). It is very slightly soluble in dimethyl sulfoxide (DMSO) and anhydrous methanol, but is sparingly soluble in these solvents when trifluoroacetic acid (0.1%) is added. (Solubility descriptions are in accordance with USP definitions).

### II. Method of Manufacture

#### *Introduction*

The chemical process used for the synthesis of CY-1899 employs chemistry which is compatible with N-protected fluorenylmethyloxycarbonyl-amino acids (Fmoc-AA). The Fmoc protecting group is easily removed under very mild conditions using piperidine. The C-terminal amino acid is attached, via a trifluoroacetic acid (TFA) sensitive ester linkage, to a moiety which is covalently attached to a polystyrene resin. Amino acid residues with potentially reactive side chains are blocked with protecting groups that are removable at the end of the synthesis by the action of TFA. Hence, cleavage and deprotection are carried out, simultaneously, at the conclusion of the synthesis.

Specifically, the synthesis of CY-1899 is performed on an Advanced ChemTech ACT200 (Louisville, KY) solid-phase peptide synthesizer using coupling cycles that are optimized for the production of this peptide product. Briefly, Fmoc-Val-resin (scale of production = 3 - 10 mmol) is introduced into a silanized glass reaction vessel. The resin is first washed with N-methylpyrrolidone (NMP), followed by removal of the N-terminal Fmoc protecting group using piperidine. During this procedure, in preparation for the coupling of the next amino acid, the next Fmoc-amino acid required in the synthesis is converted into the activated 1-hydroxybenzotriazolyl ester (Fmoc-AA-OBt) form, in a separate vessel, by reaction with 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) in dimethyl acetamide (DMA) or NMP. The activated Fmoc-amino acid ester is then transferred to the reaction vessel, and the coupling reaction is allowed to proceed. The use of NMP or DMA as solvent is founded on the basis of its ability to solubilize all of the Fmoc-amino acids and activated esters used in this synthesis, as well as its propensity to break up secondary structure in the elongating peptide chain. The latter characteristic is helpful for the successful assembly of a peptide of the length of CY-1899.

After the coupling reaction, the resin is sampled and tested with ninhydrin or tetrachloro-*p*-benzoquinone reagent to assay for completion. This assay is capable of detecting 0.2% of unreacted amino group. If the coupling reaction is incomplete (less than 99.5%), a second coupling reaction is performed. If coupling is satisfactory then the peptide chain is capped by reaction with acetic anhydride. This will ensure that any resin bound peptide chain that was not coupled with the last amino acid will be terminated. These terminated chains will then be removed at the final purification step of the bulk drug substance due to their different physical properties from CY-1899. This ensures a high



degree of sequence fidelity of CY-1899. Upon completion, the cycle is repeated until all of the amino acid constituents of CY-1899 are introduced. The palmitoyl groups are then introduced in a similar fashion.

5 The peptide is cleaved from the solid support, and all side chain protecting groups are removed by reacting a dried resin with TFA-water.

### *Synthesis*

1) General: The manufacture of peptide CY-1899 as a bulk drug substance is carried out on an Advanced ChemTech ACT 200 Peptide Synthesizer as described in the Manufacturing Formula (C9050). All equipment used in this preparation is cleaned and  
10 inspected. A Line/Equipment Clearance form is completed by Manufacturing and verified by Quality Assurance to assure that all materials and equipment are present according to specifications.

The ACT 200 Peptide Synthesizer is loaded with methylene chloride, N-methyl pyrrolidone, and isopropyl alcohol. Four liters of freshly prepared 25%  
15 piperidine in dimethylformamide in a 4 L bottle is also loaded into the ACT 200 Peptide Synthesizer.

All gas, liquid delivery and waste lines of the ACT 200 Peptide Synthesizer are cleaned and inspected. Calibrations of liquid delivery volumes are performed before synthesis.

20 The peptide is produced by solid-phase synthesis by assembly from the C-terminus to the N-terminus on a polystyrene support. The resin which has immobilized fluorenylmethyloxycarbonyl-valine (Fmoc-valine) is weighed out; to give the desired batch size from 3 - 10 mmol of amino acid which is dependent upon the degree of substitution. The substitution level is determined by first removing the Fmoc group; then the amino acid  
25 substitution level of the resin is assayed by the ninhydrin method. This is lot specific.

The weighed resin is placed in a 500 mL reaction vessel which has been pre-silanized according to standard written procedures. Delivery and removal of reagents are made through the top and bottom delivery lines.

2) Preconditioning the Resin: The manual mode is selected from the main  
30 menu and the wash selection is instructed to fill the reaction vessel with approximately 150 mL of N-methylpyrrolidone (NMP) and the filled vessel is sparged with nitrogen for 2-3 minutes. The vessel is emptied at that time and the wash step repeated twice more. This is done to precondition the resin to the solvent.

3) Deprotection: The delivery line is washed with 50 mL of NMP and then 110 mL of 25% piperidine in NMP is added to the reaction vessel and mixed with the resin for 5 minutes by sparging nitrogen through the solution. At that time the reaction vessel is emptied. Then 110 mL of 25% piperidine in NMP is added and mixed with the resin for 25 minutes with nitrogen sparging. This effects the removal of the Fmoc protecting group from the immobilized amino acid to prepare for coupling of the next residue. The resin in the reaction vessel is then washed twice consecutively with 150 mL of NMP for 2 minutes and 150 mL of isopropanol (IPA) for 2 minutes. A final 3 washes with 150 mL of NMP for 2 minutes ensures removal of piperidine. Finally the delivery line to the reaction vessel is washed with 50 mL of NMP. The efficiency of the Fmoc deprotection of the resin is then determined for all terminal amino acids, except proline, by a qualitative ninhydrin assay. When the terminal amino acid is proline, a qualitative assay with tetrachloro-*p*-benzoquinone is conducted.

4) Activation: The Fmoc-amino acid to be coupled to the immobilized acceptor is converted to an active ester. The active ester used in the synthesis of CY-1899 is the hydroxybenzotriazole ester. For example, in the first coupling step 6-20 mmol of Fmoc-serine(tBu)-OH is mixed in a second glass reaction vessel with a 6-20 mmol of N-hydroxybenzotriazole and 30-40 mL of dimethylacetamide (DMA) or NMP (depending upon the amino acid) by vortexing until a complete solution is obtained. Six to twenty mmol of coupling reagent, diisopropylcarbodiimide is added and this mixture is vortexed for 2-3 minutes. The resulting mixture is left to react for an additional 20-25 minutes.

5) Coupling: The active ester solution is now added to the thoroughly washed free amino resin for coupling. The resulting mixture is agitated and sparged with nitrogen to ensure mixing, for 2-4 hours to complete coupling. After 2-4 hours the resin is washed three times for 2 minutes with 150 mL of IPA, and washed three times for 2 minutes with 150 mL of dichloromethane (DCM) to remove all reactants. At this time the resin is analyzed for coupling efficiency by a quantitative ninhydrin or tetrachloro-*p*-benzoquinone assay. The assay involves reacting a known amount of the resin with ninhydrin in the presence of phenol. Spectroscopic analysis of the supernatant allows for quantitative determination of amines present. If the coupling efficiency is determined to be less than 99.5% then coupling of that residue is repeated. If coupling is found to be greater than or equal to 99.5%, the next step is conducted to effect capping of any residual free amine groups present.

6) Capping: The next step in the synthesis is conducted to react trace amounts (<1%) of free amino groups that may be present. This will ensure that any resin bound peptide chain that was not coupled with the last amino acid will be terminated. These terminated chains will then be removed at the final purification step of the Bulk Drug Substance due to their different physical properties from CY-1899. A fresh solution of 10% acetic anhydride in DMA is prepared and approximately 50-100 mL is added to the washed resin in the reaction vessel. This mixture is agitated for 35 minutes with nitrogen sparging. After agitation the reaction vessel is emptied and the resin is washed two times each with 150 mL of IPA, DCM, and NMP consecutively. This step completes one cycle of coupling. The first coupling step gives Fmoc-Ser(tBu)-Val-resin. This procedure is then repeated for the addition of the next amino acid residue in the sequence. This is continued until the final amino acid is added.

7) Palmitoylation: The last coupling is the addition of two palmitic acid groups to the terminal lysine. Before adding the palmitic acid groups to the tridecapeptide, 100 to 300 mg of the peptide bearing resin is retained for cleavage to give a sample for amino acid sequencing. In a separate reaction vessel, a 4-fold excess of palmitic acid is reacted with a 2-fold excess diisopropylcarbodiimide in 70 mL of DCM and 15 mL of NMP to give the anhydride. This mixture is vortexed for 2 minutes and then left for 18-23 minutes. At that time the solution of palmitic anhydride is added to the resin and coupling carried out as described above. After coupling of palmitic acid is complete the IPA and DCM washing steps are carried out as described above. The efficiency of coupling of palmitic acid is determined as described above. If the degree of coupling is determined to be less than 99.5%, recoupling is carried out. This completes the chemical assembly of the peptide on the resin. The next step is cleavage of the peptide from the resin and deprotection of the side chain residues of the peptide.

8) Drying of Resin-Bound CY-1899 Palmitoylated Peptide: At this point in the synthesis the resin is removed from the reaction vessel with the aid of 200 - 400 mL of DCM and transferred to a beaker. The resin is filtered on a sintered glass frit with suction. The moist resin is transferred to a beaker which is placed in a desiccator, and the resin is kept under vacuum until dry. At that time the resin is weighed. Typically > 32 grams of resin is obtained.

9) Cleaving and Drying of CY-1899 Palmitoylated Peptide Powder Intermediate: The peptide bearing resin is now ready for cleavage to yield the peptide.

Protecting groups on side chain functionalities are also removed during cleavage from the resin. A cleavage solution of 95% aqueous trifluoroacetic acid is prepared and cooled to 0-4°C for 20-30 minutes. The resin to be cleaved is cooled to 0-4°C in a reactor and 300-600 mL of cleavage solution is added. The cleavage reactor is removed from the cooling bath and stirred at ambient temperature for 1.5 hours. At that time the resin is removed by filtration through a glass frit. The filtrate is placed in a flask and the solvent removed by evaporation under reduced pressure. Approximately 20 grams of the Palmitoylated Peptide Powder Intermediate are obtained from reaction of 6 mmol of valine resin as described above. After sampling, this material is ready for final purification. A reserve sample is also taken.

A 50 mL polypropylene centrifuge tube holding the Palmitoylated Peptide Powder Intermediate (unpurified) is labeled and stored at -70°C in a secured area until use.

### III. Purification

#### *Introduction*

CY-1899 Palmitoylated Peptide Intermediate, which is an unpurified drug substance, is purified by taking advantage of the differential solubility of CY-1899 and its related impurities in 50% acetic acid/water. CY-1899 Palmitoylated Peptide Intermediate is insoluble in 50% acetic acid/water while many of the impurities in CY-1899 Palmitoylated Peptide Intermediate are readily soluble and can be removed by washing with 50% acetic acid/water. Figure 1 outlines the process steps in the purification of CY-1899 drug substance.

#### *Manufacture of CY-1899 Bulk Drug Substance*

The material described above is suspended at  $10 \pm 5$  mg/mL in 2-8°C 50% acetic acid/water. The suspension is homogenized with a Brinkman Polytron unit for a total of  $16 \pm 5$  minutes at 25,000 RPM. The insoluble CY-1899 peptide is recovered by centrifugation at 10,000 X g for  $30 \pm 15$  minutes. This procedure is repeated an additional 4 times for a total of five 50% acetic acid/water washes of the CY-1899 Palmitoylated Peptide Intermediate.

Following the last wash with 50% acetic acid/water, the CY-1899 Palmitoylated Peptide Intermediate is washed twice with water for irrigation to reduce the amount of residual acetic acid and allow efficient lyophilization. The pellet from the 50%

acetic acid/water wash is resuspended in an equal volume of water for irrigation and homogenized for 2-4 minutes to thoroughly resuspend the pellet. The insoluble CY-1899 Palmitoylated Peptide is recovered by centrifugation for  $60 \pm 30$  minutes at 10,000 X g. This water wash is repeated for a total of two washes with water for irrigation.

5           The final pellet containing CY-1899 Palmitoylated Peptide is resuspended in an appropriate volume of water for irrigation and lyophilized. This lyophilized powder is CY-1899 Bulk Drug Substance.

          This CY-1899 Bulk Drug Substance may be formulated immediately following appropriate in-process tests or stored at  $-70^{\circ}\text{C}$ . The Bulk Drug Substance is  
10       stored in a glass or polypropylene container. The container is sealed in a mylar pouch containing a drying agent and stored at  $-70^{\circ}\text{C}$ .

#### IV. Impurities

          The purity of CY-1899 is determined by high performance liquid  
15       chromatography (HPLC). The purity of the CY1899 bulk drug substance as compared to the unpurified palmitoylate peptide powder is shown in Figures 2 (unpurified) and 3 (purified).

          Ion spray high resolution mass spectrometry of CY-1899 Bulk Drug  
20       Substance indicates the presence of numerous N-acetylated, truncated derivatives of the tridecapeptide (Figure 4). These peptides are formed as a result of the capping procedure used during peptide synthesis.

          Semipreparative HPLC has enabled isolation of material very much enriched  
25       in several of the impurities present in the Bulk Drug Substance. Preliminary high resolution ion spray mass spectrometry (Figure 5) of these materials suggests the presence of some CY-1899 degradation products (possibly from the dehydration of a glutamine or asparagine side chain) as well as several impurities with molecular weights higher than that of CY-1899.

#### V. Dosage Forms

30       CY-1899 is provided in vials as a sterile, concentrated solution of CY-1899 in 99.9% DMSO/0.1% TFA. Two concentrations are available: 10 mg/mL and 20 mg/mL. A reconstitution diluent (PBS [phosphate buffered saline], 2.5 mL/vial) and a

dilution diluent (10% DMSO/0.01% TFA in PBS, 1.8 mL/vial) are provided for use in preparation of the final dosage form.

The final dosage form is prepared immediately prior to administration by mixing the sterile CY-1899 solution with the reconstitution diluent to yield a sterile suspension for injection. The two dosage forms are described below. If necessary, the final dosage form suspension may be further diluted (with the dilution diluent) to obtain the desired dose.

Dosage Form A: CY-1899 Suspension for Injection, 1 mg/mL

CY-1899 concentrated solution: CY-1899 2 mg/0.2 mL/vial (10 mg/mL)

When reconstituted with 1.8 mL of PBS, the final dosage form suspension contains:  
CY-1899 1 mg/mL in 10% DMSO/0.01% TFA

Dosage Form B: CY-1899 Suspension for Injection, 5 mg/mL

CY-1899 concentrated solution: CY-1899 8 mg/0.4 mL/vial (20 mg/mL)

When reconstituted with 1.2 mL of PBS, the final dosage form suspension contains:  
CY-1899 5 mg/mL in 25% DMSO/0.025% TFA

The remainder of this section contains information regarding the formulation and method of manufacture of the following clinical supplies:

CY-1899 Final Vial Product (Sterile Concentrate)

2 mg/0.2 mL/vial (10 mg/mL)

8 mg/0.4 mL/vial (20 mg/mL)

Sterile Diluent for Reconstitution of CY-1899 Concentrates

Phosphate Buffered Saline (2.5 mL/vial)

Sterile Diluent for Dilution of CY-1899 Suspension

PBS, 10% DMSO, 0.01% TFA (1.8 mL/vial)

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

5

## WHAT IS CLAIMED IS:

1. A method of preparing a purified synthetic lipopeptide, the method comprising:  
contacting a composition comprising the synthetic lipopeptide with an  
5 aqueous solution, such that impurities are selectively dissolved; and  
separating the lipopeptide from the aqueous solution to obtain a purified  
lipopeptide.
- 10 2. The method of claim 1, wherein the aqueous solution comprises an  
organic acid.
3. The method of claim 2, wherein the organic acid is acetic  
acid.
- 15 4. The method of claim 3, wherein the aqueous solution is 50% acetic  
acid/50% water.
- 20 5. The method of claim 1, wherein the steps of contacting and  
separating are repeated 5 times.
6. The method of claim 1, wherein the step of separating is carried out  
by centrifugation.
- 25 7. The method of claim 7, wherein the lipopeptide comprises two lipid  
residues.
8. The method of claim 7, wherein the lipid residue is a palmitoyl  
residue.
- 30 9. The method of claim 1, wherein the lipopeptide comprises between  
about 25 and about 35 amino acid residues.



10. The method of claim 1, wherein the lipopeptide comprises an amino acid sequence comprising an CTL epitope from hepatitis B virus core antigen.

11. The method of claim 10, wherein the CTL epitope is Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val.

12. The method of claim 1, wherein the lipopeptide comprises a T helper epitope.

13. The method of claim 12, wherein the T helper epitope is Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu.

14. The method of claim 1, wherein the lipopeptide is (PAM)<sub>2</sub>-Lys-Ser-Ser-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val.

15. A composition consisting essentially of a solvent and a lipopeptide having the formula:

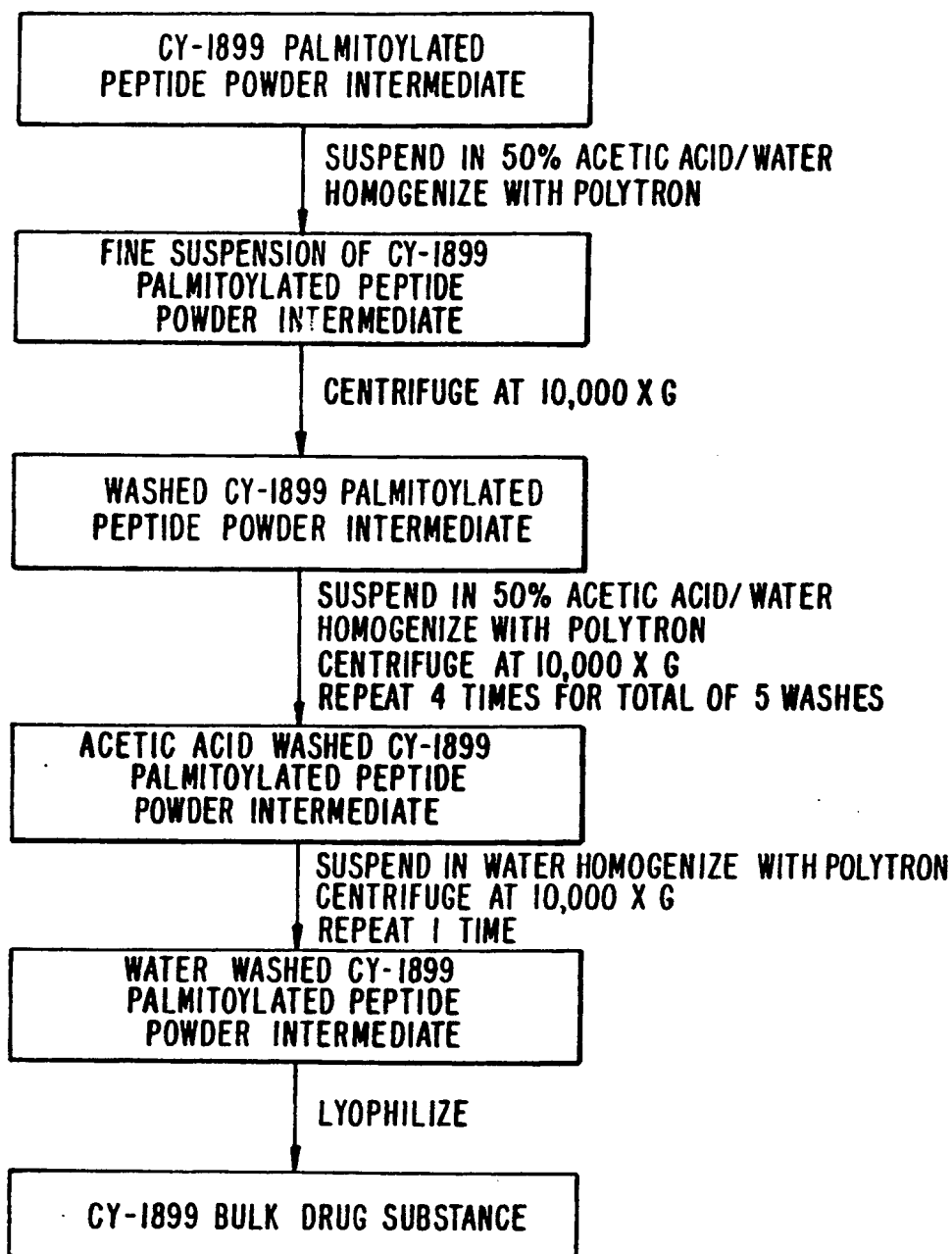
(PAM)<sub>2</sub>-Lys-Ser-Ser-Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu-Ala-Ala-Ala-Phe-Leu-Pro-Ser-Asp-Phe-Phe-Pro-Ser-Val.

16. The composition of claim 15, wherein the solvent is 99.9% DMSO/0.1% TFA.

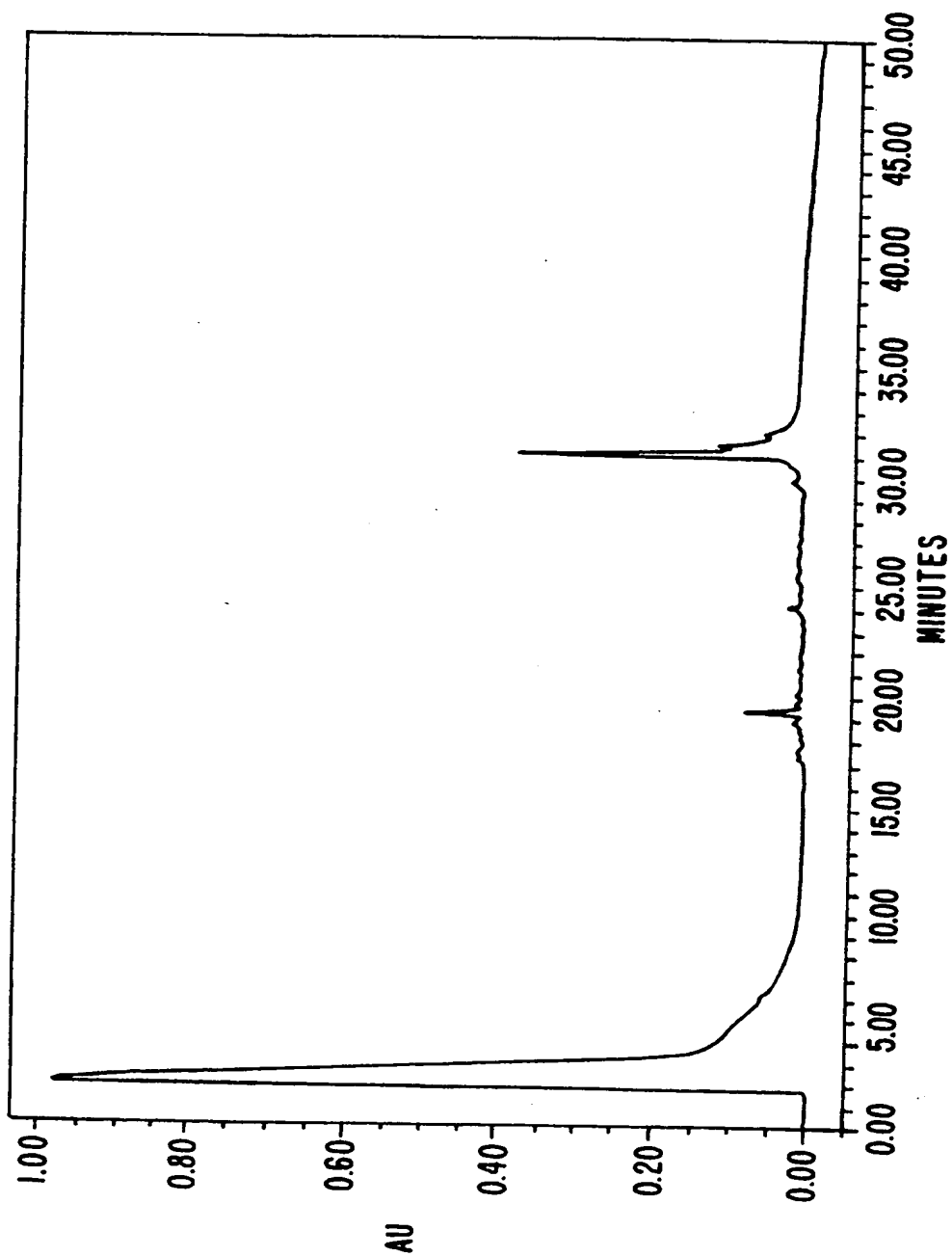
17. The composition of claim 15, wherein the lipopeptide is present at a concentration of about 10mg/ml.

18. The composition of claim 15, wherein the lipopeptide is present at a concentration of about 20mg/ml.

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**FIG. 1.**

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*FIG. 2.*

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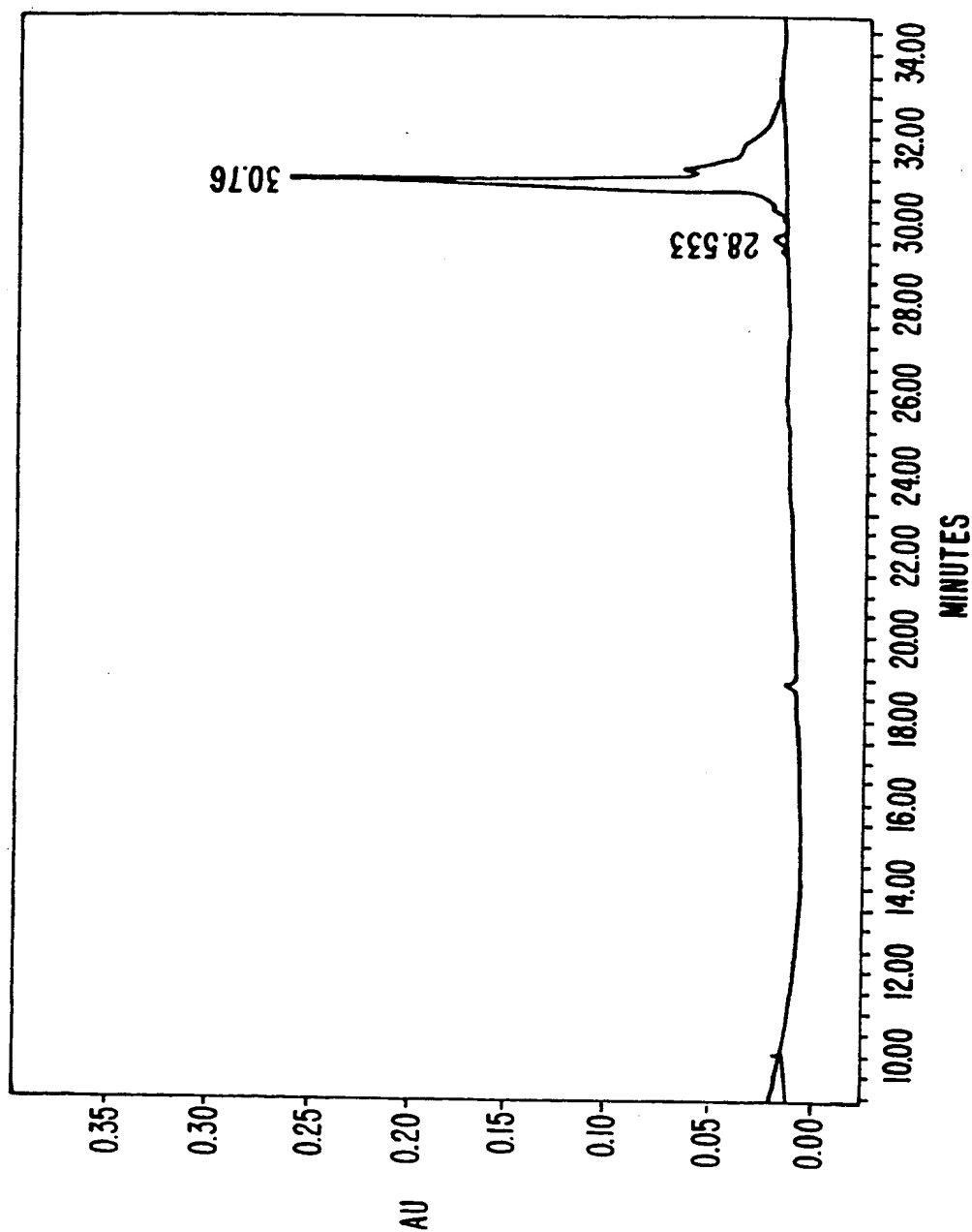


FIG. 3.

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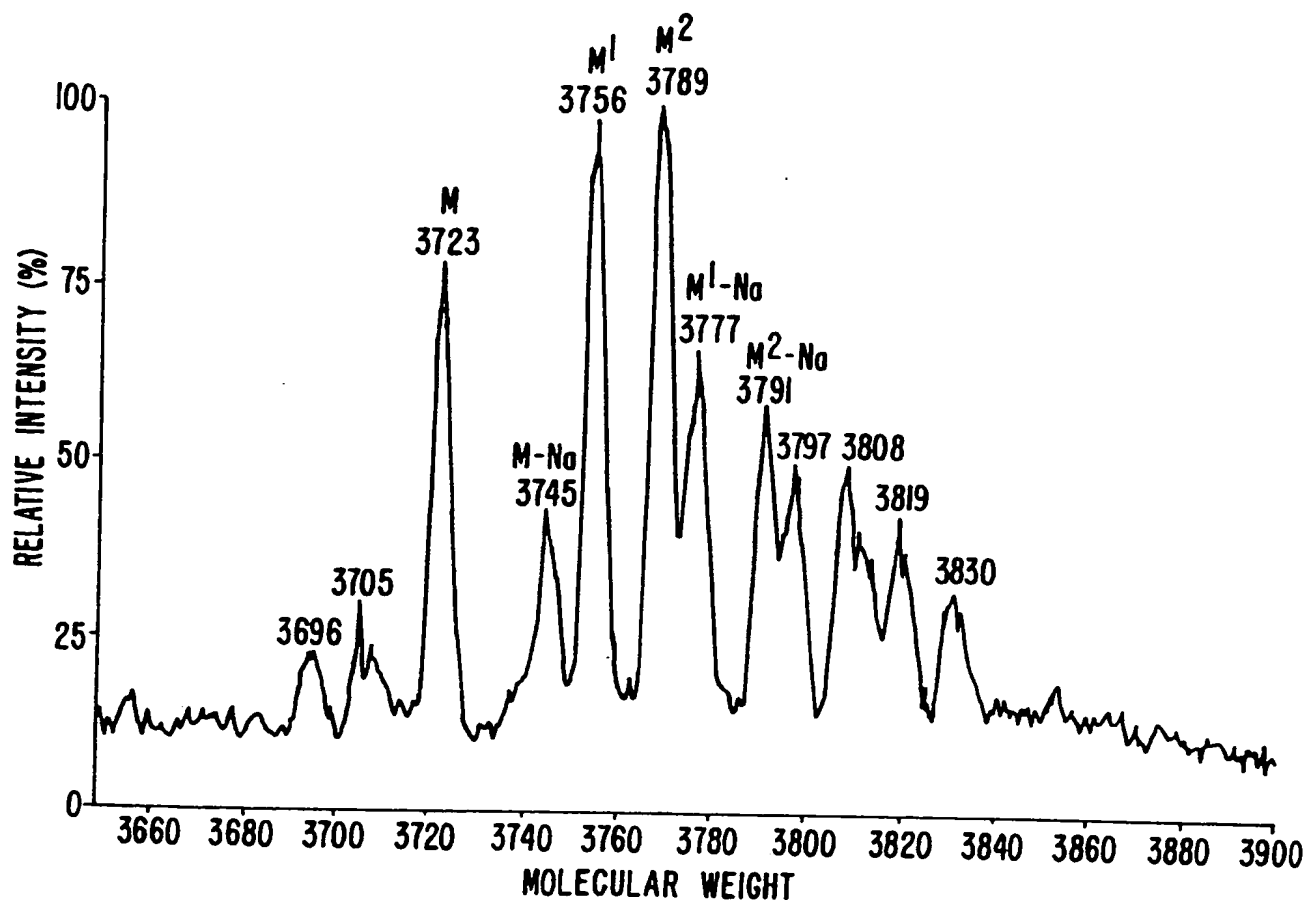


FIG. 4.

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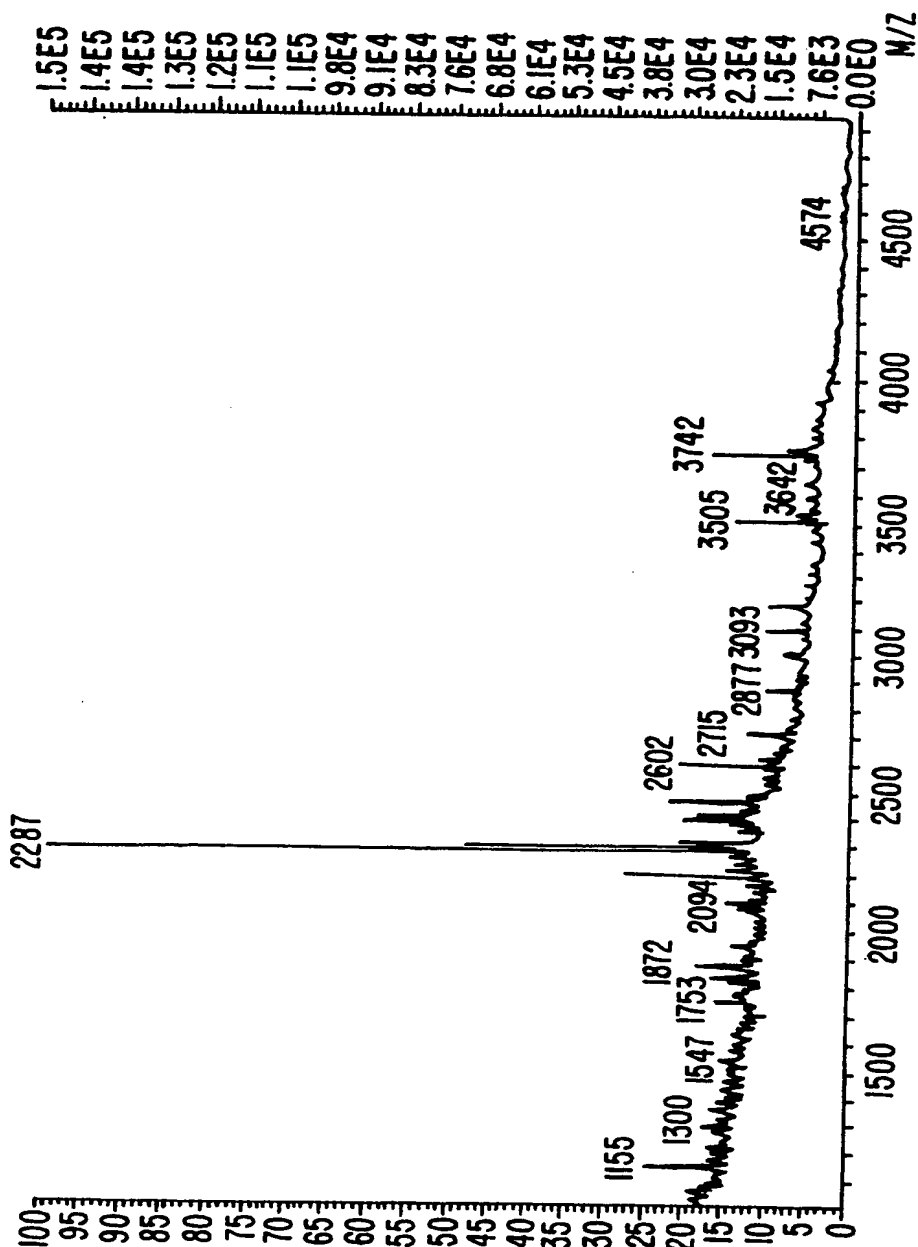


FIG. 5.

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09579

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/16; C07K 1/14, 1/30

US CL : 530/324, 418, 412; 514/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/324, 418, 412; 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

search terms: lipopeptide, purification, acetic acid

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 4,665,053 A (ROBERT et al.) 12 May 1987 (12/05/87), see entire document, especially columns 6-10.	1-3 ----- 1-3, 5, 7-14
Y	VITIELLO et al. Development of a Lipopeptide-based Therapeutic Vaccine to Treat Chronic HBV Infection. The Journal of Clinical Investigation. January 1995, Vol. 95, No. 1, pages 341-349.	1-3, 5, 7-18
X	US 4,666,886 A (BASCHANG et al.) 19 May 1987 (19/05/87), see entire document, especially column 29, lines 1-38.	1-2

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 SEPTEMBER 1996

Date of mailing of the international search report

30 SEP 1996

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